

Distinct Pattern of Commensal Gut Microbiota in Toddlers with Eczema

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Key Words

Allergy · *Bifidobacterium* · Eczema · *Enterococcus* · Gut microbiota · Hygiene hypothesis

Abstract

Background: Recent studies have demonstrated differences in the composition of gut microbiota in infants with and without allergic diseases, particularly eczema. **Methods:** A case-control study involving 21 toddlers (age 3.0 ± 0.5 years) with and 28 age-matched toddlers without eczema was conducted. Four groups of aerobic gut microbiota were identified and quantitated in stool samples grown on selective media. Three groups of anaerobes were enumerated by fluorescent in situ hybridization followed by quantitative flow cytometry. We also performed molecular typing of lactic-acid-producing bacteria (LAB) and enterococcal isolates to facilitate detailed analysis at species level by bacterial 16S rDNA sequencing. **Results:** Toddlers with eczema harbored significantly lower counts of *Bifidobacterium* [(median 0.14 (25th and 75th percentile: 0.04 and 0.47) vs. 0.71% (0.16, 1.79) of cells acquired, $p = 0.003$)] and *Clostridium* [(0.28 (0.09, 0.78) vs. 0.83% (0.35, 1.82) of cells acquired, $p = 0.012$)] but significantly higher counts of total LAB [7.3 (6.1, 8.5) vs. 5.7 (4.4, 7.3) log CFU/g, $p = 0.006$] in par-

ticular enterococci [6.3 (4.8, 7.4) vs. 5.0 (3.4, 6.4) log CFU/g, $p = 0.018$]. There was no significant correlation between eczema severity score and bifidobacterial counts. **Conclusion:** The results further confirm previous reports that the gut microecosystem differs between children with and without eczema and extend them beyond infancy.

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Introduction

It has been proposed that an altered lifestyle associated with industrialization over the last several decades has modified exposure to microbes. A disturbed intestinal microecosystem early in life impairs normal development of the immune system, and this in turn has been suggested to influence host susceptibility to allergy [1].

During recent decades, the prevalence of atopic dermatitis (AD) has increased considerably worldwide [2]. However, the prevalence of atopy, including AD, is lower in children with an anthroposophical lifestyle, suggesting that non-hereditary factors may play a critical role in the manifestations of various allergic diseases [3].

Gut microbiota are essential for the maintenance of intestinal homeostasis, and their influence may extend

beyond the gut, modifying systemic immunity [4, 5]. However, the roles played by various components of the gut microbiota are still unknown. Their potential role in the development of atopy is of interest, as gut microbiota may provide a basis for future allergy-preventive strategies. In this respect, *Bifidobacterium* and other probiotic bacteria have attracted attention. Lower *Bifidobacterium* counts have been reported in subjects with AD [6]. In a recent study, Young et al. [7] have suggested that the beneficial effect of *Bifidobacterium* may be strain dependent. However, a significant proportion of the gut microbiota comprises microorganisms that cannot be isolated, and their influence on the immune system is unknown. We have compared the composition of certain gut microbiota in urban Singapore toddlers with and without eczema, employing conventional as well as modern microbiology methods and molecular typing of lactic-acid-producing bacteria (LAB) and enterococcal isolates.

Patients and Methods

Study Design

The study groups comprised 21 toddlers with eczema and 28 age-matched healthy controls. The diagnosis of eczema was made by two pediatricians (B.W.L. and H.P.v.B.) based on diagnostic criteria described by Hanifin and Rajka [8]. A simple severity score, the Three Item Severity Score (TIS), was employed to assess the severity of skin symptoms. The TIS score is based on the evaluation of erythema, edema/papulation and excoriation on a scale from 0 to 3, with a total of 9 as the maximum score [9]. Healthy controls were recruited from a larger random cohort being followed for breastfeeding habits at the National University Hospital, Singapore. They had neither signs of eczema or of other allergic diseases at the time of recruitment nor a personal or family history of eczema, allergic rhinitis or asthma. Furthermore, children were excluded if they had an acute illness or had taken any medicines, including antibiotics, within 14 days of stool collection. The study was approved by the Institutional Review Board at the National University Hospital.

Clinical Characteristics of the Participants

All toddlers were 3.0 ± 0.5 years. The median TIS score for affected toddlers with onset of eczema in infancy was 4 (range 3–7). All children were born in Singapore and none were receiving probiotic supplementation. Our subjects represent a cohort of children living in a cosmopolitan society consuming a diet intermingled with a mixture of Malay, Chinese, Indian and European cultures. The eczema and healthy groups were also similar in previous exposure to antibiotics (eczema 82% vs. healthy 80%). Eczematous subjects (72%) and healthy subjects (57%) were exclusively breast-fed, for mean durations of 4.39 vs. 2.21 months, respectively (unpaired t test, $p = 0.14$). There was 1 subject in the eczema group who was on a cow's milk exclusion diet due to cow's milk allergy. All subjects had birth weights above 2.5 kg except for 1 subject in the healthy group with a birth weight of 1.9 kg.

Sample Collection and Preparation

Fecal samples were collected into sterile plastic vials by parents, stored at -20°C and delivered to the laboratory within 20 h. The samples were all kept cool on a dry ice pack during transport and upon arrival to the laboratory immediately diluted with saline to make up a 0.1 g/ml concentration. After homogenizing, conventional culture and fixation were performed.

Culture

The bacterial counts and identification of the various species were based on growth, color and number of colonies on selective media, Gram staining and cell morphology. In a preliminary study, API-kit-based biochemical tests were performed to confirm the selectivity of media. Selective media used were de Man-Rogosa-Sharpe agar (MRS; Oxoid) for total LAB; Slanetz and Bartley agar (S&B; Oxoid) for *Enterococcus* spp.; McConkey purple agar (Oxoid) for coliforms and *Staphylococcus* Medium No. 110 (Oxoid) for *Staphylococcus* spp. The samples were serially diluted (10^{-1} to 10^{-8}) in saline in a bio-safety cabinet. The MRS agar plates were incubated at 37°C , 5% CO_2 for 48 h, McConkey purple agar plates at 35°C aerobically, overnight, S&B agar plates at 35°C for 2 h and then at 44°C for 44 h and *Staphylococcus* No. 110 agar plates at 35°C aerobically for 48 h. Bacteria were quantified employing the drop plate technique by culturing 25 μl of sample in duplicate per dilution on the same plate.

Fluorescence in situ Hybridization (FISH)

For anaerobic bacteria, a selective count was done using 16S-rRNA-specific probes and FISH. The optimum probe concentration was found to be 5 ng/ μl , as described in [10]. Specific probes used were CAT-CCG-GCA-TTA-CCA-CCC for *Bifidobacterium*; GAG-CCG-CAA-ACT-TTC-ACAA for the *Bacteroides fragilis* subgroup; CCG-CCT-GCC-TCA-AAC-ATA for the *Bacteroides distasonis* subgroup; TTA-TGC-GGT-ATT-AAT-CT(C/T)-CCT-TT for the *Clostridium histolyticum* subgroup, and GTT-ATC-CGT-GTG-TAC-AGGG for the *Clostridium lituseburense* subgroup [10, 11].

These probes were tested with the following control bacteria by means of flow cytometry as well as fluorescent microscopy and found to be specific. The positive controls were *Bacteroides* and *Bifidobacterium* strains provided by the Department of Microbiology, National University of Singapore. *C. lituseburense* ATCC25759 and *C. histolyticum* ATCC17859 were obtained from American Type Culture Collection, USA. A strain of *Enterococcus faecalis* provided by the Department of Microbiology, National University of Singapore, was used as a negative control. The specificity of probes for the FISH analysis was validated by hybridization with their positive control strains as well as cross-hybridized with negative control strains. In pilot experiments with samples from 5 children, we also obtained reasonable counts expressed in percent of FITC-labeled cells from those fixed stool samples. In almost all samples, all targeted bacteria groups could be detected, employing the protocol.

For FISH analysis, 0.01 g of stool sample was fixated as described by Langendijk et al. [10]. Briefly, 1 ml was removed from the 10^{-1} dilution and centrifuged at 12,000 rpm for 3 min to pellet cells. Cells were then fixed overnight with 1 ml 4% paraformaldehyde (vol/vol) at 4°C , washed twice in PBS and resuspended in 1 ml of PBS:ethanol (1:1/ v/v). After storing at -20°C for at least 2 h, subsamples of the fixed cells were hybridized overnight at 50°C

in hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl) with its FITC-labeled specific probe(s). Hybridization buffer used for detecting *Clostridia* spp. was supplemented with 10% dextran sulfate. After overnight hybridization, cells were washed twice with PBS before quantification by flow cytometry.

Samples were analyzed with a Becton-Dickinson FACScalibur flow cytometer (San Jose, Calif., USA). Cells hybridized with labeled probes were compared with cells incubated with hybridization buffer alone. Ten thousand cells were acquired for analysis per sample and bacterial concentrations adjusted to lower than 1,000 events/s. True-positive counts were determined by subtracting background shift observed in unstained samples.

Genotypic Characterization of LAB

Molecular genotyping was done as described by Randazzo et al. [12]. Briefly, bacterial genomic DNA was isolated and amplification of the conserved bacterial 16S rDNA region was performed to produce a 1.5-kb DNA fragment, followed by restriction digestion using three enzymes to generate restriction fragment length polymorphism (RFLP) patterns. Selected pure strains were then partially sequenced in order to search for homology with currently available sequences.

Ten colonies each from S&B and MRS agar were selected from 7 children (3 healthy controls and 4 patients with eczema) and further characterized. The sample from 1 of the healthy subjects had no colonies on S&B agar, giving a total of 130 colonies (60 from S&B and 70 from MRS). Samples from children with and without eczema were compared with regard to diversity of MRS-derived LAB isolates and S&B-derived enterococcal isolates, and genotypic characteristics of coccal isolates from MRS with those from S&B. Furthermore, strains were identified by partial sequencing of the conserved region of bacterial 16S rDNA.

For isolation of genomic DNA from individual strains of bacteria, colonies were selected from each selective medium plate according to colony morphologies and depending on the frequency of occurrence on selective medium. The cell cultures (1.5 ml) were centrifuged at 8,000 rpm for 10 min, and the cell pellets were washed and resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Using a 2-ml tube containing 0.3 g of sterile 0.1-mm glass beads, the mixture was vortexed for 10 min. The homogenate was centrifuged at 13,000 rpm for 5 min and the supernatant fluid was isolated. The supernatant was purified using columns from the Qiagen DNeasy DNA isolation kit. Supernatant fluid was stored at -20°C until use.

PCR amplification was done using primers 7f 5'-AGAGTTT-GATC/TA/CTGGCTCAG-3' and 1510rev 5'-ACGG(C/T)TAC-CTTGTTACGACTT-3'. Thermal cycles were programmed as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, 52°C for 30 s and 68°C for 1.5 min, and, finally, 68°C for 7 min.

Restriction enzyme digestion of 16S rDNA PCR products was performed with *Hae*III, *Hin*6I and *Alu*I (MBI Fermentas). DNA gel electrophoresis of the digested products was performed on 2% (w/v) agarose gel.

Partial sequence of selected pure strains was directly sequenced using primer 1,100 reverse [12] 5'-GGGTTGCGCTCGTTG-3'. Sequence analysis was done using BigDye version 3.1 terminator cycle (Applied Biosystems, Foster City, Calif., USA). Homology searches of 16S rDNA sequences were performed using the BMCI BLAST service available on the World Wide Web. (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

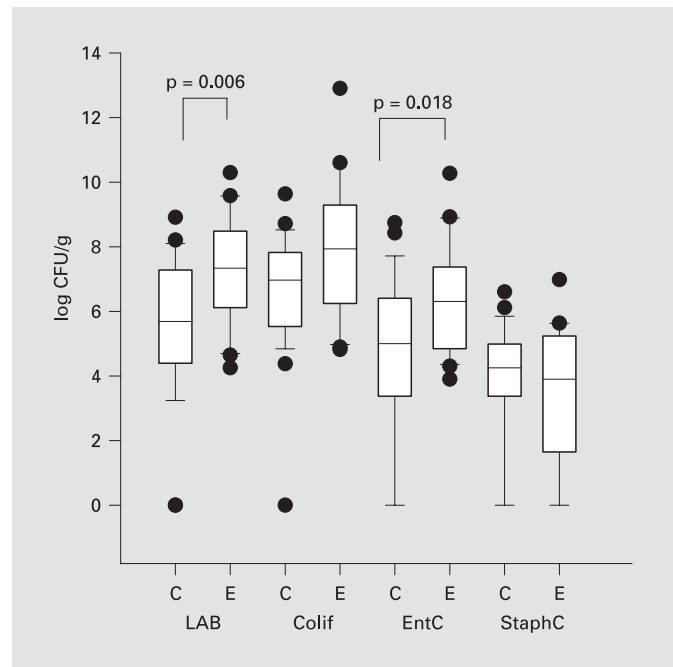


Fig. 1. Aerobic counts in eczematous (E, n = 21) and healthy (C, n = 28) toddlers determined by conventional culture (log CFU/g). Colif = Coliforms; EntC = *Enterococcus* spp.; StaphC = *Staphylococcus* spp. The 10th, 25th, 50th, 75th and 90th percentiles are displayed and statistically significant p values are given.

Statistics

Aerobic counts were expressed in log CFU/g of wet fecal samples. The detection limit was 3.6. The FISH counts were expressed as the percentage of FITC-positive cells from a total of 10,000 cells. Since the bacterial counts were not normally distributed, the Mann-Whitney U test was employed for analysis. All counts were expressed as medians (lower and upper quartiles). Fischer's exact test was used to compare the prevalence of colonization. Spearman's rank correlation was used to compare numerical correlations between species. All statistical analysis was done using computer software SPSS. Significance was set at $p < 0.05$.

Results

Bacterial Counts (Culture/FISH)

Toddlers with eczema harbored significantly higher counts of LAB [7.3 (6.1, 8.5) vs. 5.7 log CFU/g (4.4, 7.3), $p = 0.006$], in particular enterococci [6.3 (4.8, 7.4) vs. 5.0 log CFU/g (3.4, 6.4), $p = 0.018$]. In addition, coliform counts tended to be higher in the eczema group than in children without eczema [7.9 (6.2, 9.3) vs. 7.0 log CFU/g (5.5, 7.8), $p = 0.084$; fig. 1]. In contrast, markedly less *Bifidobacterium* spp. [0.14 (0.04, 0.47) vs. 0.71% of cells

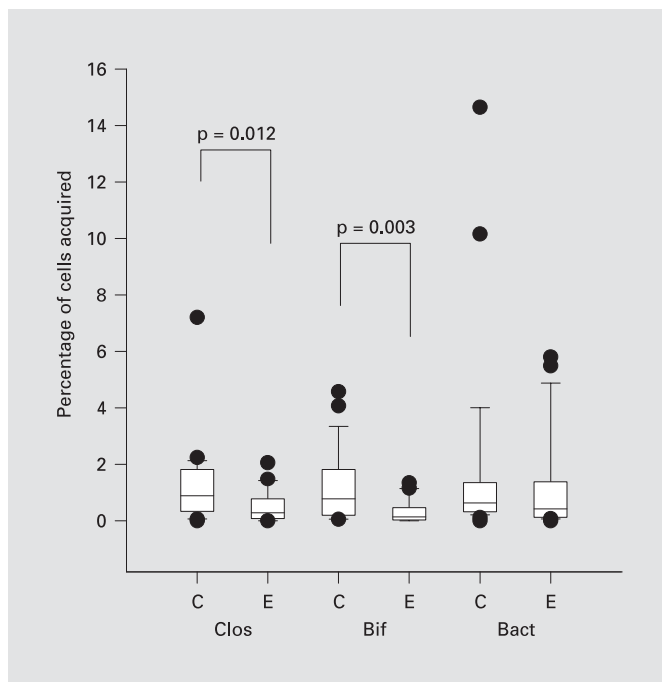


Fig. 2. Anaerobic counts in eczematous (E, n = 21) and healthy (C, n = 28) toddlers determined by FISH, expressed as a percentage of cells acquired. Clos = *Clostridium* spp; Bif = *Bifidobacterium* spp.; Bact = *Bacteroides* spp. The 10th, 25th, 50th, 75th and 90th percentiles are displayed and statistically significant p values are given.

acquired (0.16, 1.79), $p = 0.003$] and *Clostridium* spp. [0.28 (0.09, 0.78) vs. 0.83% of cells acquired (0.35, 1.82), $p = 0.012$] were found in toddlers with eczema (fig. 2). The *Staphylococcus* and *Bacteroides* counts were similar in the two groups.

Prevalence of *Bifidobacterium* (Culture/FISH)

Although not statistically significant, toddlers with eczema tended to be less often colonized with *Bifidobacterium* (86 vs. 100%, $p = 0.072$) compared with the healthy toddlers.

Correlation of Various Bacterial Counts

A negative correlation between *Staphylococcus* and *Clostridium* and a positive correlation between the anaerobes (*Bifidobacterium*, *Clostridium* and *Bacteroides*) were observed in healthy children (table 1). In contrast, only a positive correlation between *Bifidobacterium* and coliforms was observed in children with eczema (table 1). In addition, a positive correlation was seen between counts of total LAB and enterococci as well as *Bifidobacterium* and *Bacteroides* in both groups of children.

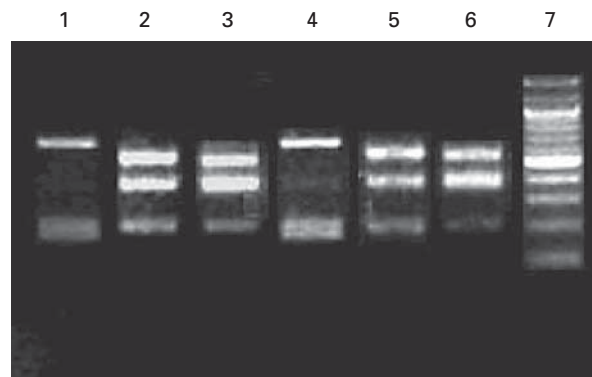


Fig. 3. Predominant RFLP pattern of enterococcal colonies isolated. Lanes 1–3 = Amplified 16S rDNA of a coccal isolate from MRS digested with restriction enzymes *AluI*, *HaeIII* and *Hin6I*, respectively; lanes 4–6 = amplified 16S rDNA of an enterococcal isolate from S&B medium digested with *AluI*, *HaeIII* and *Hin6I*, respectively; lane 7 = 100-bp marker.

Genotypic Characterization of LAB

RFLP of 16S bacterial rDNA demonstrated that the enterococcal isolates could occasionally be found growing on selective media of LAB (fig. 3). A predominant enterococcal RFLP pattern, also shown in figure 3, represented 82% of the isolates (49/60 colonies, range 40–100%). This pattern was observed in both groups of children. Since significantly higher enterococcal counts were observed in the eczema group, we then identified the enterococcal strains commonly found by partial sequencing of the conserved 16S rDNA region. Twelve strains showing the predominant RFLP pattern (2 colonies isolated from each of the 6 toddlers) were partially sequenced. Irrespective of the host eczema status, half of these isolates were identified as *E. faecalis* V583 and the rest as *Enterococcus faecium* Efae02_10. All of them shared 98–100% homology compared to the control strains.

Discussion

This is the first study relating pediatric gut microbiota to AD in the tropical region of South East Asia. The differences in the composition of fecal microbiota between children with and without eczema substantiate previous observations in infancy and extend them into toddlers [1, 13–16]. Furthermore, this study observed reduced and different patterns of interbacterial relationships in children with eczema compared to healthy controls (table 1).

Table 1. Relationships between different species of bacteria in healthy individuals and children with eczema

	LAB	Coliforms	<i>Enterococcus</i>	<i>Staphylococcus</i>	<i>Clostridium</i>	<i>Bifidobacterium</i>	<i>Bacteroides</i>
Healthy children							
LAB		NS	0.711*	NS	NS	NS	NS
Coliforms			NS	NS	NS	NS	NS
<i>Enterococcus</i>				NS	NS	NS	NS
<i>Staphylococcus</i>					-0.499*	NS	NS
<i>Clostridium</i>						0.661*	0.56*
<i>Bifidobacterium</i>							0.75*
<i>Bacteroides</i>							
Children with eczema							
LAB		NS	0.499*	NS	NS	NS	NS
Coliforms			NS	NS	NS	0.636*	NS
<i>Enterococcus</i>				NS	NS	NS	NS
<i>Staphylococcus</i>					NS	NS	NS
<i>Clostridium</i>						NS	NS
<i>Bifidobacterium</i>							0.555*
<i>Bacteroides</i>							

Correlations are expressed by Spearman's ρ . Significant correlations are indicated. * $p < 0.01$, NS indicates no significant correlation.

This observation further demonstrates that the patterns of gut microbiota differ between children with and without eczema. A lower rate of colonization with *Bifidobacterium* through the 1st year of life has been reported in infants who subsequently develop allergic manifestations [13]. Watanabe et al. [6] compared fecal microbiota between 30 eczematous and 68 healthy subjects (age 3–13 years) by conventional culture and found higher bifidobacterial counts in the latter. Through a culture-independent whole-cell hybridization method, we also demonstrated a reduction in the bifidobacterial flora in eczematous subjects, substantiating the notion that *Bifidobacterium* is associated with protection against allergic diseases.

We observed higher enterococcal and LAB counts in our eczema subjects compared to controls. Microscopic examination revealed that the majority of our LAB isolates in this study were either *Lactobacillus*-like or *Enterococcus*-like bacteria. The presence of enterococcal colonies in both S&B (highly selective) and MRS media likely contributes to the correlation between *Enterococcus* and LAB counts in our subjects. Our PCR-RFLP studies on enterococcal colonies from both S&B and MRS media further substantiate that S&B-derived enterococci were also present in MRS-derived total LAB counts. Taken together, the data indicate that the higher LAB counts in toddlers with eczema was indeed due to elevated enterococcal counts.

It is, however, not feasible to correct LAB counts for enterococci due to the differential selection properties of enterococci by S&B and MRS, and therefore the absolute *Lactobacillus* counts could not be evaluated.

In previous studies, skin symptoms of infants with atopic eczema have been shown to improve significantly after intervention with a probiotic strain of *Lactobacillus* [17, 18]. In contrast, it has been reported that enterococci less often colonize the gut of young infants (at 1 week and 1 month) who later develop allergy compared to those who did not [13]. Interestingly, Shimada et al. [19, 20] reported that lysed *E. faecalis* strain [FK-23 (LFK)] could downregulate eosinophilia and IgE-mediated allergy in mice. In a subsequent clinical trial, the strain was shown to improve clinical symptoms of allergic rhinitis in a pilot study comprising 50 patients [21].

These findings suggest that some *Lactobacillus/Enterococcus* strains exhibit the potential to relieve clinical symptoms of allergic diseases. Mohamed et al. [22] compared *E. faecalis* isolates from subjects with and without endocarditis and observed significant differences in the degree of biofilm formation between isolates from the two origins. This exciting observation suggests that enterococcal isolates from different disease states exhibit different biochemical abilities and may thus exert different impacts on their hosts. Two enterococcal strains, *E. faecalis*

V583 and *E. faecium* Efae02_10, were found to be predominant enterococcal species in the gut of Singaporean children. Total enterococcal counts were found to be higher in children with eczema. If confirmed in subsequent studies, our data indicate that the immunological roles of *Enterococcus* may not be uniform for all strains. As our observation was based on microbial cultures (excludes unculturable bacteria), and given the constant debate on the contrasting roles of *Enterococcus* in health and disease, the role of *Enterococcus* in the development of allergy deserves further study.

In addition, Kalliomäki et al. [14] conducted a study involving 76 infants at high risk of atopic diseases who later became allergic or healthy children, but failed to show any significant difference in terms of their *Lactobacillus*/*Enterococcus* counts. Similarly, Watanabe et al. [6] also failed to display any difference in the two counts between eczematous and healthy subjects. Again, we demonstrate that eczematous children are not deficient in lactic-acid producing aerobic species, e.g. *Lactobacillus* and *Enterococcus*. This observation is noteworthy and further explorations are needed, as they were previously thought to be key contributing species to lowering the prevalence of atopic diseases [23]. Young et al. [7] reported that fecal samples from Ghana, a country with a low incidence of allergy, almost always contained *Bifidobacterium infantis* whereas those from areas with a high prevalence of allergy did not. Moreover, it has been revealed that certain *Lactobacillus* strains influence B cells [24] and dendritic cells [25] in a strain-dependent manner. Hence, the impact of a bacterial species on immunity may not be uniform throughout the genus. These findings imply that allergic children may be lacking particular beneficial strains rather than the entire genus. Hence, the use of probiotics in specific health applications is a more complex issue than we previously thought.

A high detection rate of *Clostridium difficile* was reported in allergic infants through an indirect method comparing microflora-associated characteristics in feces [26]. Kalliomäki et al. [14] described a reduced ratio of *Bifidobacterium* to *Clostridium* as a characteristic of 3-week gut microflora for high-risk neonates who later developed atopic sensitization at 12 months. In the same study, atopic subjects had higher counts of *C. histolyticum* (as determined by FISH), but there was no difference in the detection of cultured *C. difficile*. Furthermore, a prospective study by Björkstén et al. [13] showed higher counts of clostridia in allergic infants only at 3 months of age, but not later. In contrast, Watanabe et al. [6] reported lower cultured clostridial counts in eczema subjects,

though this was not statistically significant. However, a study in gnotobiotic mice proved that colonization with *Clostridium* could promote the development of the intestinal immune system [27]. In our study, we employed the FISH technique targeting two gut clostridial groups, i.e. *C. histolyticum* and *C. lituseburense*, and found them to be significantly lower in children with eczema. These conflicting reports on *Clostridium* may be related to the different species targeted in various studies.

Our findings further substantiate the presence of significant differences in certain species of microbiota in eczema subjects. The composition of most human microbiota is still unknown, however, and so far the only consistent difference between allergic and non-allergic children seems to be a lower prevalence and/or lower counts of *Bifidobacterium* in the former. Studies in mice suggest that microbiota disruption could drive the development of airway allergic response without previous systemic antigen priming [28]. This observation was consistent for different antigens and mouse strains [29]. Thus, there are both animal experiments and clinical studies indicating that gut microbiota may play a key role early in life. Our study suggests that the role of microbiota in childhood allergy extends beyond infancy.

Acknowledgments

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References

- Björkstén B, Naaber P, Sepp E, Mikelsaar M: The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* 1999;29:342–346.
- Schultz Larsen F, Hanifin JM: Secular change in the occurrence of atopic dermatitis. *Acta Derm Venereol Suppl* 1992;176:7–12.
- Alm JS, Swartz J, Lilja G, Scheynius A, Pershagen G: Atopy in children of families with an anthroposophic lifestyle. *Lancet* 1999;353:1485–1488.
- Cebra JJ: Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* 1999;69:1046S–1051S.
- Rhee KJ, Sethupathi P, Driks A, Lanning DK, Knight KL: Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J Immunol* 2004;172:1118–1124.
- Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri Y, Kumemura M: Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J Allergy Clin Immunol* 2003;111:587–591.
- Young SL, Simon MA, Baird MA, Tannock GW, Bibiloni R, Spencely K, Lane JM, Fitzharris P, Crane J, Town I, Addo-Yobo E, Murray CS, Woodcock A: Bifidobacterial species differentially affect expression of cell surface markers and cytokines of dendritic cells harvested from cord blood. *Clin Diagn Lab Immunol* 2004;11:686–690.
- Hanifin J, Rajka G: Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl* 1980;92:44–47.
- Wolkerstorfer A, de Waard van der Spek FB, Glazenburg EJ, Mulder PG, Oranje AP: Scoring the severity of atopic dermatitis: three item severity score as a rough system for daily practice and as a pre-screening tool for studies. *Acta Derm Venereol* 1999;79:356–359.
- Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MH, Welling GW: Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl Environ Microbiol* 1995;61:3069–3075.
- Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW: Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 1998;64:3336–3345.
- Randazzo CL, Restuccia C, Romano AD, Caggia C: *Lactobacillus casei*, dominant species in naturally fermented Sicilian green olives. *Int J Food Microbiol* 2004;90:9–14.
- Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M: Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;108:516–520.
- Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E: Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001;107:129–134.
- Kirjavainen PV, Apostolou E, Arvola T, Salminen SJ, Gibson GR, Isolauri E: Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. *FEMS Immunol Med Microbiol* 2001;32:1–7.
- Kirjavainen PV, Arvola T, Salminen SJ, Isolauri E: Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? *Gut* 2002;51:51–55.
- Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S: Probiotics in the management of atopic eczema. *Clin Exp Allergy* 2000;30:1604–1610.
- Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E: Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001;357:1076–1079.
- Shimada T, Cheng L, Ide M, Fukuda S, Enomoto T, Shirakawa T: Effect of lysed *Enterococcus faecalis* FK-23 (LFK) on allergen-induced peritoneal accumulation of eosinophils in mice. *Clin Exp Allergy* 2003;33:684–687.
- Shimada T, Cheng L, Yamasaki A, Ide M, Motonaga C, Yasueda H, Enomoto K, Enomoto T, Shirakawa T: Effects of lysed *Enterococcus faecalis* FK-23 on allergen-induced serum antibody responses and active cutaneous anaphylaxis in mice. *Clin Exp Allergy* 2004;34:1784–1788.
- Shimada T, Cheng L, Enomoto T, Yang X, Miyoshi A, Shirakawa T: Lysed *Enterococcus faecalis* FK-23 oral administration reveals inverse association between tuberculin responses and clinical manifestations in perennial allergic rhinitis: a pilot study. *J Invest Allergol Clin Immunol* 2004;14:187–192.
- Mohamed JA, Huang W, Nallapareddy SR, Teng F, Murray BE: Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect Immun* 2004;72:3658–3663.
- Alm JS, Swartz J, Björkstén B, Engstrand L, Engstrom J, Kuhn I, Lilja G, Mollby R, Norin E, Pershagen G, Reinders C, Wreiber K, Scheynius A: An anthroposophic lifestyle and intestinal microflora in infancy. *Pediatr Allergy Immunol* 2002;13:402–411.
- Ibnou-Zekri N, Blum S, Schiffrin EJ, von der Weid T: Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties in vitro. *Infect Immun* 2003;71:428–436.
- Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, Zaat BA, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML: Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* 2005;115:1260–1267.
- Böttcher MF, Nordin EK, Sandin A, Midtvedt T, Björkstén B: Microflora-associated characteristics in faeces from allergic and nonallergic infants. *Clin Exp Allergy* 2000;30:1590–1596.
- Umesaki Y, Setoyama H, Matsumoto S, Imaoka A, Itoh K: Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. *Infect Immun* 1999;67:3504–3511.
- Noverr MC, Noggle RM, Toews GB, Huffnagle GB: Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect Immun* 2004;72:4996–5003.
- Noverr MC, Falkowski NR, McDonald RA, McKenzie AN, Huffnagle GB: Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. *Infect Immun* 2005;73:30–38.